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26. (New) A bispecific antibody comprising the peptide according to Claim 23.

N.E

27. (New) A recombinant antibody product comprising the peptide according to Claim 23.--

THE REMARKS

The Amendments

The specification is amended on page 1 to recite the priority claim, and to recite the correct address of the American Type Culture Collection.

The specification is amended on page 6 to recite "(SEQ ID NO: 7)" in order to identify the SEQ ID NO. of the DNA sequence.

Claim 1 is amended to recite "A recombinant antibody product, comprising the V_H domain of the OKT3 antibody, wherein the cysteine at position H100A of said V_H domain is substituted with a polar amino acid , wherein said position H100A is according to the Kabat numbering system". Support for the amendment is found, for example, at page 2, lines 9-14.

Claim 2 is amended to recite "The recombinant antibody product". Support for the amendment is found, for example, at page 2, lines 9-14 and Claims 1 and 2 as originally filed.

Claim 3 is amended to recite "comprising the amino acid sequence depicted by SEQ ID NO: 2". Support for the amendment is found, for example, at page 5, lines 15-16.

Claim 4 is amended to recite "A method for the production of the recombinant antibody product according to any one of claims 1 to 3, characterized by the steps of: a) obtaining mRNA from freshly subcloned hybridoma cells of OKT3 and transcription into cDNA, b) amplifying the DNA coding for the variable domains of the light and heavy chains by means of PCR, c) cloning of the DNA obtained in b) into a vector adapted for site-specific mutagenesis as well as introduction of a mutation in said position H100A of said VH domain, wherein said position H100A is according to the Kabat numbering system, wherein said mutation is the substitution of a cysteine with a polar amino acid, and d) inserting the mutated DNA obtained in c) in an expression vector and expression in a suitable expression system." Support for the amendment is found, for example, in Claim 4 as originally filed.

Claims 6-9 are amended to in order that Claims 6-9 depend only from Claim 4.

Support for the amendment is found, for example, in Claims 6-9 as originally filed.

Claim 7 is amended to recite "said cloning uses a primer comprising the sequence depicted by SEQ ID NO: 7". Support for the amendment is found, for example, in page 3, line 12.

New Claims 12-22 are supported by Claims 3-9 as originally filed.

New Claim 23 is supported by page 5, lines 15-16.

New Claim 24 is supported by page 2, lines 4-6 and page 5, lines 15-16.

New Claim 25 is supported by page 4, lines 24-26.

New Claim 26 is supported by page 4, lines 11-13.

New Claim 27 is supported by page 4, lines 11-19 and 24-26, and page 5, lines 15-16.

No new matter is added in any of the above amendment and the Examiner is respectfully requested to enter the amendments and reconsider the application.

The Response

1. Objection to specification

The Examiner objects to the lack of a first sentence claiming priority to a prior application. Applicants have amended the specification by inserting a sentence to the beginning of the specification to indicate priority claimed to International Application No. PCT/DE98/01409, filed May 22, 1998, and German Patent Application No. 187 21 700.1, filed May 23, 1997. Therefore, in view of the amendment, the Examiner's objection should be withdrawn.

The Examiner objects to the alleged lack of an abstract. Applicants traverse this objection. Applicants respectively point out that page 10 contains a separate sheet upon which is the abstract of the disclosure. Therefore, the Examiner's objection should be withdrawn.

The Examiner objects to the lack of a SEQ ID NO. on page 6, line 18. Applicants have amended the specification by inserting a SEQ ID NO. to identify the DNA sequence.

Therefore, in view of the amendment, the Examiner's objection should be withdrawn.

The Examiner objects to the specification in that the address of the American Type Culture Collection is not current. Applicants have amended the specification by replacing the disclosed address with the current address. Therefore, in view of the amendment, the Examiner's objection should be withdrawn.

The Examiner objects to the phrase "by means of PCR" in Claim 4. Applicants traverse this objection in that "amplifying [a DNA] by means of PCR is not atypical claim language. PCR is a well-known molecular biology technique to one of ordinary skill of the art, and it is clear and definite that the amplifying of the DNA is by the technique of PCR. Therefore, the Examiner's objection should be withdrawn.

The Examiner objects to Claims 4-9 as being in improper form because a multiple dependent claim cannot depend from a multiple dependent claim. Applicants have amended these claims so that no multiple dependent claim depends from a multiple dependent claim. Therefore, in view of the amendments, the Examiner's objection should be withdrawn.

The Examiner objects to Claims 1, 3, 5, and 7 for failing to recite the SEQ ID NOs. Applicants have amended Claims 3 and 7 to recite the appropriate SEQ ID NOs. The term "OKT3" is a proper noun of a specific "monoclonal IgG 2a-type antibody originating from mice, which recognizes an epitope of an ε-subunit of the human CD3 complex" and the hybridoma which produces OKT3 is deposited with American Type Culture Collection (page 1, lines 5-16). Claim 5 recites the primers of Bi5, Bi8, Bi4 and Bi3f. "Bi5", "Bi8", "Bi4", and "Bi3f" specifically denote a specific primers of specific sequences known to one skilled in the art as described in Dübel, et al. (*J. Immunol. Meth.* 175:89-95, 1994) and Gotter, et al. (*Tumor Targeting* 1:107-114, 1995) (page 2, lines 15-32). Therefore, in view of the amendment, the Examiner's objection should be withdrawn.

2. 35 U.S.C. § 112, second paragraph rejections.

The Examiner rejects Claims 1-9 under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicants regard as the invention. The rejection of Claims 1-9 is traversed in part and overcome in part in view of the amendments.

A) The Examiner rejects Claims 1-9 in that allegedly the term "OKT3" is a trademark or trade name. Applicants respectfully traverse this basis of rejection. The term "OKT3" is not a trademark or trade name. The term "OKT3" is a proper noun of a specific "monoclonal IgG 2a-type antibody originating from mice, which recognizes an epitope of an ε-subunit of the human CD3 complex" (page 1, lines 5-7). Therefore amended Claims 1-9 are clear and definite.

B) The Examiner rejects Claims 1-9 in that allegedly the phrase "position H100A of the OKT3 antibody" is indefinite in that the disclosure does not provide a numbered reference sequence and that the term "OKT3" is a trademark. Applicants respectfully traverse this basis of rejection in part and avoid the rejection by amendment in part. For the reasons given above, the term "OKT3" is not a trademark. Amended Claim 1 recites "wherein said position H100A is according to the Kabat numbering system". Therefore amended Claims 1-9 are clear and definite.

C) The Examiner rejects Claim 1-9 in that allegedly that it is unclear if position H100A of the OKT3's antibody has the cysteine to serine exchange on one or both heavy chains. Amended Claim 1 recites "the V_H domain of the OKT3 antibody, wherein the cysteine at position H100A of said V_H domain is substituted with a polar amino acid". It is clear that the position H100A relates to only one V_H domain. Therefore amended Claim 1 is clear and definite.

D) The Examiner rejects Claims 2 and 3 in that allegedly there is insufficient antecedent basis for "The monoclonal antibody" and "the polar amino acid". Amended Claim 1 recites

"A recombinant antibody product" while amended Claims 2 and 3, which depend from Claim 1, recite "The recombinant antibody product". There is antecedent support for "The recombinant antibody product" in Claim 1. Amended Claim 1 recites "a polar amino acid" while amended Claim 2, which depends from Claim 1, recites "the polar amino acid". There is antecedent support for "the polar amino acid" in Claim 1. Therefore amended Claims 2 and 3 are clear and definite.

E) The Examiner rejects Claims 4-9 in that allegedly there are essential steps missing from Claims 4-9 needed for the production of the monoclonal antibodies of any of Claims 1-3. Amended Claim 4, from which Claims 5-9 depend from, recites the production of a "recombinant antibody product". Since a single-chain Fv is sufficient to constitute a "recombinant antibody product", the claimed method is clear and definite in the describing a method to produce a "recombinant antibody product". Therefore amended Claims 4-9 are clear and definite.

F) The Examiner rejects Claims 4 and 6-9 in that allegedly the term "suitable primers" is indefinite. Amended Claim 4 has the term "suitable primers" deleted. Claims 6-9 depend from Claim 4 and do not recite "suitable primers". Therefore amended Claims 4 and 6-9 are clear and definite.

G) The Examiner rejects Claims 4-9 in that allegedly the term "the desired mutation" is indefinite. Amended Claim 4 has the term "the desired mutation" deleted. Claims 5-9 depend from Claim 4 and do not recite "the desired mutation". Therefore amended Claims 4-9 are clear and definite.

H) The Examiner rejects Claims 8 and 9 in that allegedly the term "pHOG21" is indefinite because the characteristics of "pHOG21" are not known. Applicants respectfully traverse this basis of rejection. The term "pHOG21" specifically denotes a distinct plasmid as described in Figure 1 (page 4, line 32 to page 5, line 14). In addition, Kiripyanov, et al. (*J. Immunol. Meth.* 196:51-62, 1996) describes pHOG21 on Figure 1 (cited on page 3, lines 25-27), and

Kiripyanov, et al. (*J. Immunol. Meth.* 200:69-77, 1997; a copy of which is attached) describes pHOG21 on Figure 1 (page 70-71, "Vector constructions"). These references further demonstrate that the term "pHOG21" specifically denotes a distinct plasmid, and that one skilled in the art by the disclosure of the present application would know that pHOG21 clearly and definitively describes a distinct plasmid. Therefore Claims 8 and 9 are clear and definite.

For the reasons above, the § 112, second paragraph rejection of Claims 1-9 should be withdrawn.

3. 35 U.S.C. § 112, first paragraph, enablement rejection.

The Examiner rejects Claims 8 and 9 under 35 U.S.C. § 112, first paragraph because allegedly "the pHOG21 vector is required to practice the claimed invention". Applicants traverse this rejection .

MPEP 2164.4 states:

> "In order to make a rejection, the examiner has the initial burden to establish a reasonable basis to question the enablement provided for the claimed invention. *In re Wright*, 999 F.2d 1557, 1567, 27 USPQ2d 1510, 1513 (Fed. Cir.) 1993) (examiner must provide a reasonable explanation as to why the scope of protection provided by a claim is not adequately enabled by the disclosure)."

The Examiner has not established a reasonable basis to question the enablement provided for Claims 8 and 9. The Examiner states that: "It is apparent that the pHOG21 vector is required to practice the claimed invention. As a required element, it must be known *and readily available to the public or obtainable by a repeatable method set forth in the specification.*" (page 6, lines 14-16; emphasis in the original). The Examiner has not stated that "the pHOG21 vector" is not enabled by the disclosure of the present application.

However, if the Examiner alleges that the pHOG21 vector is not enabled by the disclosure of the present application, then Applicants respectfully contend that the specification, when filed, contained sufficient information to enable one skilled in the art to make and use the invention, including the expression vector pHOG21. The specification discloses the individual components of plasmid pHOG21 in Figure 1 (page 4, line 32 to page 5, line 14), and that plasmid pHOG21 is also taught in Kiripyanov, et al. (*J. Immunol. Meth.*

196:51-62, 1996). Therefore, the specification enables the making and using of plasmid pHOG21.

For the reasons stated above, this 35 U.S.C. § 112, first paragraph rejection of Claims 8 and 9 should be withdrawn.

The Examiner rejects Claims 4-9 under 35 U.S.C. § 112, first paragraph because allegedly the specification does not enable "the desired mutation". Applicants avoid this rejection in that Claim 4 as amended does not recite "the desired mutation".

Therefore, this 35 U.S.C. § 112, first paragraph rejection of Claims 4-9 should be withdrawn.

4. 35 U.S.C. § 102(b)

The Examiner rejects Claims 1-9 under 35 U.S.C. § 102(b) as being anticipated by Kipriyanov, et al. (*Protein Engineering* 10(4):445-53, 1997). The Applicants respectively traverse this rejection because Kipriyanov, et al. (1997) is not a prior art reference.

Applicants submit a correspondence from the Production Editor of the Oxford University Press indicating that the *Protein Engineering* Volume 10, No. 4, 1997 issue was dispatched on June 2, 1997. The priority date of the present application is May 23, 1997, which predates the date on which this reference was dispatched. Since the priority date of the present application (May 23, 1997) clearly predates the publication date of Kipriyanov, et al. (1997) (a date later than June 2, 1997), therefore **Kipriyanov, et al. (1997) is not a printed publication in this or a foreign country more than one year prior to the date of the present application**. Hence, Kipriyanov, et al. (1997) is not a prior art reference under 35 U.S.C. § 102(b).

For the reason stated above, the 35 U.S.C. § 102(b) rejection of Claims 1-9 over Kipriyanov, et al. (1997) should be withdrawn.

5. 35 U.S.C. § 103(a)

The Examiner rejects Claims 1-9 under 35 U.S.C. § 103(a) as being obvious over Kroon, et al. (*Pharmaceutical Res.* 9:1386-93, 1992) in view of Kipriyanov, et al (*J. Immunol.*

Meth. 196:51-62, 1996) and in further view of Senoo, et al. (U.S. Patent No. 5,852,1767). Applicants respectively traverse the rejection of Claims 1-9 because Kroon, et al. in view of Kipriyanov, et al (1996) and in further view of Senoo, et al. do not render obvious Claims 1-9.

Kroon, et al. suggest that, among a considerable number of "candidate" amino acids, degradative changes in the cysteine of the third CDR of OKT3 may have a significant impact on the binding affinity of the antibody. However, Kroon, et al. neither disclose nor provide evidence that this is a primary event responsible for the instability of OKT3 at low or high temperatures. For example, extensive deamidations of several asparagine residues were found and five methionine residues were oxidized. These degradative changes were found both in the constant domains and in the antibody variable domains. It is a well known fact to a person skilled in the art that the mutation of only one amino acid can potentially alter the structure of a protein. Particularly critical amino acids can be recognized by the fact that they have remained unchanged during evolution. Kroon, et al., for example, point out in the last paragraph that the conserved residue Ans386 may be a source of instability in other antibodies. Whereas changes in the structure of a protein through the degradation of one or more amino acids may perhaps lead to a more exposed position of oxidation-susceptible amino acids such as methionine and cysteine, however, Kroon, et al. do not disclose whether any one amino acid can be responsible for such instability. Thus, whereas Kroon, et al. disclose that several amino acids are subject to degradation an storage of OKT3, it is not obvious to one skilled in the art which one amino acid can be primarily responsible for the instability.

Senoo, et al. showed that the substitution of one or more particular cysteines by serine improved the stability of basic fibroblast growth factor under acidic conditions. However, this is a completely different protein to OKT3. It can be substantially mutated without losing activity. For example, as many as 46 amino acid residues were deleted in a preferred mutant of this protein. Radic, et al. (*Methods* 11:20-26, 1997; a copy of which is attached) show that changing a single amino acid of an anti dsDNA antibody (R53 of VH3H9) can completely eliminate binding (see page 20, "Abstract"). In the present case, the mutated cysteine in OKT3 is situated in the center of the CDR3 domain of the variable heavy chain domain, which many structural studies have shown to play a major role in antigen binding. Even

minor changes in this region would be expected to have a profound affect on the antibody binding activity.

Thus is no reasonable expectation of success that substituting the cysteine of the V_H domain of the OKT3 antibody at position H100A, according to the Kabat numbering system, with a polar amino acid would increase its stability at antibody binding but that it still binds antigen with no appreciable loss if binding activity.

For the reasons stated above, the 35 U.S.C. § 103(a) rejection of Claims 1-9 over Kroon, et al. in view of Kipriyanov, et al (1996) and in further view of Senoo, et al. should be withdrawn.

CONCLUSION

In view of the foregoing amendments and remarks, the Applicants believe the application is in good and proper condition for allowance. Early notification of allowance is earnestly solicited. If, in the opinion of the Examiner, a telephone conference would expedite the prosecution of the subject application, the Examiner is encouraged to call the undersigned at (650) 463-8109. A telephone conference is especially requested if the Examiner intends to maintain the present rejections.

Respectfully submitted,

Date: October 9, 2001

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VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the Specification

On page 1, after "Mutated OKT3 Antibody", insert:

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application is a National Stage of International Application No. PCT/DE98/01409, filed May 22, 1998, which claims priority to German Patent Application No. 197 21 700.1, filed May 23, 1997.

Paragraph beginning at line 5 of page 1 has been amended as follows:

OKT3 is a monoclonal IgG 2a-type antibody originating from mice, which recognizes an epitope of an -subunit of the human CD3 complex (Kung et al., Science 206, pp. 347-349 (1979); Van Wauwe, et al., J. Immunol. 124, pp. 2708-2713 (1980); Transy et al., Eur. J. Immunol. 19, pp. 947-950 (1989)). The method of obtaining the monoclonal antibody from the corresponding hybridoma is described in detail in these publications. Furthermore, the OKT3-producing hybridoma cell line was deposited by the owner of European patent 0 018 795 under ATCC No. CRL 8001 with the American Type Culture Collection, [12301 Parklawn Drive, Rockville, MD, 20852] 10801 University Boulevard, Manassas, VA 20110-2209, on April 26, 1979. OKT3 has been used for a long time to suppress a T-cell response thus preventing the rejection of transplants (Thistlethwaite et al., Transplantation 51, pp. 1207-1212 (1991)). On the other hand, OKT3 can also trigger T-cell activation and proliferation, which stimulates the effector cells, which can be used for the adoptive cancer immunotherapy (Yannelly et al., J. Immunol. Meth. 1, pp. 91-100 (1990)). OKT3 was used as such and as a component of a bispecific antibody to direct cytotoxic T-lymphocytes against tumor cells or virus-infected cells (Nitta et al., Lancet 335, pp. 368-376 (1990); Sanna et al., Bio/Technology 13, pp. 1221-1224 (1995)). Furthermore, humanized versions of the OKT3-monoclonal antibody which were expressed in COS cells are also known (Woodle et al., J. Immunol. 148, pp. 2756-2763 (1992); Adair et al., Human. Antibod. Hybridomas, pp. 41-47 (1994)). So far there has been the problem that OKT3 has no

sufficient stability and particularly cannot be expressed in known recombinant expression systems in stably fashion and sufficient amount.

Paragraph beginning at line 10 of page 6 has been amended as follows:

Thereafter, the amplified DNA was 'blunt-end' ligated into the vector pCR-Skript SK(+) sold by the company of Stratagene, which has been cleaved using the SrfI restriction enzyme. Mutations were inserted in the V_H domain originating from OKT3 by site specific mutagenesis (Kunkel et al., Meth. Enzymol. 154, pp. 367-382 (1987)). The amino acid substitution at position H100A of OKT3 (exchange of cysteine for serine) was carried out using the primer SK1 5'-GTAGTCAAGGCTGTAATGATCATC (SEQ ID NO. 7).

In the Claims

Claims 1-4 and 6-9 are amended as follows:

1. (Amended) A [monoclonal] recombinant antibody product, [characterized by an exchange of] comprising the V_H domain of the OKT3 antibody, wherein the cysteine [for another polar amino acid] at position H100A [the OKT3 antibody known under this name] of said V_H domain is substituted with a polar amino acid , wherein said position H100A is according to the Kabat numbering system .

2. (Amended) The [monoclonal] recombinant antibody product , characterized in that the polar amino acid is serine.

3. (Amended) The [monoclonal] recombinant antibody product according to claim 1[or 2, characterized in that it includes the sequence indicated in figure 2] comprising the amino acid sequence depicted by SEQ ID NO:2.

4. (Amended) A method for the production of the [monoclonal] recombinant antibody product according to any one of claims 1 to 3, characterized by the steps of:

a) [obtainment of] obtaining mRNA from freshly subcloned hybridoma cells of OKT3 and transcription into cDNA,

b) [amplification of] amplifying the DNA coding for the variable domains of the light and heavy chains by means of PCR [using suitable primers] ,

c) cloning of the DNA obtained in b) into a vector adapted for site-specific mutagenesis as well as introduction of [the desired mutation using suitable primers,] a mutation in said position H100A of the V_H domain, wherein said position H100A is according to the Kabat numbering system, wherein said mutation is the substitution of a cysteine with a polar amino acid, and

d) [insertion of] inserting the mutated DNA obtained in c) in an expression vector and expression in a suitable expression system.

6. (Amended) The method according to claim 4 [or 5], wherein the vector used in step c) is pCR-Skript SK(+).

7. (Amended) The method according to [any one of claims 4 to 6] claim 4 , wherein [the primer SK1 5'-GTAGTCAAGGCTGTAATGATCATC is used in step c)] said cloning uses a primer comprising the sequence depicted by SEQ ID NO: 7 .

8. (Amended) The method according to [any one of claims 4 to 7] claim 4 , wherein the expression vector used in step d) is pHOG21.

9. (Amended) The method according to [any one of claims 4 to 8] claim 4 , wherein the expression takes place in XLl-Blue E. *coli* cells.

Please the following new claims:

--12. (New) The method according to claim 5, wherein the vector used in step c) is pCR-Skript SK(+).

13. (New) The method according to claim 5, wherein said cloning uses a primer comprising the sequence depicted by SEQ ID NO: 7.

14. (New) The method according to claim 6, wherein said cloning uses a primer comprising the sequence depicted by SEQ ID NO: 7.

15. (New) The method according to claim 5, wherein the expression vector used in step d) is pHOG21.

16. (New) The method according to claim 6, wherein the expression vector used in step d) is pHOG21.

17. (New) The method according to claim 7, wherein the expression vector used in step d) is pHOG21.

18. (New) The method according to claim 4, wherein the expression takes place in XLl-Blue E. *coli* cells.

19. (New) The method according to claim 5, wherein the expression takes place in XLl-Blue E. *coli* cells.

20. (New) The method according to claim 6, wherein the expression takes place in XLl-Blue E. *coli* cells.

21. (New) The method according to claim 7, wherein the expression takes place in XLl-Blue E. *coli* cells.

22. (New) The method according to claim 8, wherein the expression takes place in XLl-Blue E. *coli* cells.

23. (New) A peptide comprising the amino acid sequence depicted by SEQ ID NO:2.

24. (New) An antibody comprising the peptide according to Claim 23.

25. (New) A single-chain antibody comprising the peptide according to Claim 23.

26. (New) A bispecific antibody comprising the peptide according to Claim 23.

27. (New) A recombinant antibody product comprising the peptide according to Claim 23.--

ELSEVIER

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High level production of soluble single chain antibodies in small-scale *Escherichia coli* cultures

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Abstract

We have investigated the effect of growth and induction conditions on the production of soluble single-chain Fv antibody fragments in *Escherichia coli* under the control of wt *lac* promoter. The scFv was directed into the periplasmic space by a *pel*B leader sequence. Addition of sucrose to the medium gave a 15–25-fold increase in the yield of soluble scFv-phOx (3.0 mg/l) for bacterial shake-tube cultures and an increase of 80–150-fold (16.5 mg/l) for shake-flask cultures. Using flask culture in the presence of 0.4 M sucrose, a significant amount of scFv was released into the medium. We found that the scFv could be made to accumulate in the periplasm or be secreted into the medium by simply changing the incubation conditions and the concentration of the inducer. The ratio between soluble antibody fragments and insoluble scFv aggregates proved to be dependent on the strength of the promoter. Lowering the incubation temperature below 20°C had no effect on the yield of soluble antibody fragments in the periplasm, but they were no longer secreted into the medium. An example of high level production in shake-flask cultures and one-step purification by immobilized metal affinity chromatography (IMAC) is described for a soluble scFv specific for the T cell surface antigen CD3. The biological activity of the purified anti-CD3 scFv was demonstrated by flow cytometry. This method should be especially useful for the functional screening of a large number of clones in small-scale cultures.

Keywords: Single chain Fv antibody fragment; Small scale culture; Bacterial expression system

1. Introduction

The variable region (Fv) of an antibody comprises the antibody V_H and V_L domains and is the smallest antibody fragment containing a complete antigen binding site. To stabilize the association of the recombinant V_H and V_L domains, they have been linked in a single-chain Fv construct with a short peptide that connects the carboxy terminus of one domain and the amino terminus of the other (Bird et al., 1988; Huston et al., 1988). In comparison to the

Abbreviations: BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; FACScan, fluorescence-activated cell scanner; Fv, $V_H + V_L$ domains of an antibody; IMAC, immobilized metal affinity chromatography; IPTG, isopropyl-β-D-thio-galactopyranoside; mAb, monoclonal antibody; PBS, phosphate buffered saline; PCR, polymerase chain reaction; scFv, single-chain Fv; phOx, hapten 2-phenyloxazol-5-one; V_H, variable heavy chain domain of an antibody; V_L, variable light chain domain of an antibody.

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much larger Fab', $F(ab')_2$ and IgG forms of monoclonal antibodies, scFvs have lower retention times in non-target tissues, and exhibit more rapid blood clearance and better tumor penetration (Milenic et al., 1991; Yokota et al., 1992; Adams et al., 1993). ScFvs therefore represent valuable molecules for the targeted delivery of drugs, toxins, or radionuclides to a tumor site.

Unlike glycosylated whole antibodies, scFvs can be easily produced in bacterial cells as functional antigen binding molecules. There are two basic strategies to obtain recombinant antibody fragments from *Escherichia coli*. The first is to produce antibody proteins as cytoplasmic inclusion bodies followed by refolding in vitro (Huston et al., 1988, 1991). However, a major problem with refolding is the formation of incorrect intramolecular disulfide bridges (Buchner and Rudolph, 1991; Buchner et al., 1992). The second approach is to imitate the situation in the eucaryotic cell for secreting a fully folded antibody. In *E. coli*, the secretion machinery directs proteins carrying a specific signal sequence to the periplasm (Pugsley, 1993). A proportion of periplasmic scFv may also leak out and can be recovered from the culture medium.

Periplasmic expression has permitted the functional testing of a wide variety of antibody fragments with different antigen binding specificities (for a review, see Skerra, 1993). The scFv antibody fragments are usually correctly processed in the periplasm, they contain intramolecular disulfide bonds and are soluble (Glockshuber et al., 1990). However, a high-level expression of an scFv fragment with a bacterial signal peptide in *E. coli* often results in the aggregation of antibody fragments after transport to the periplasm (Whitlow and Filpula, 1991; Kipriyanov et al., 1994). This may possibly be caused by a high protein concentration of the scFv in the periplasmic space, which might favor the formation of insoluble aggregates over correct folding (Bowden and Georgiou, 1990; Kiefhaber et al., 1991). However, the overexpression of some enzymes of the *E. coli* folding machinery such as GroES/L chaperonins, disulfide-isomerase DSbA and prolines-*cis*-*trans*-isomerase (PPIase) do not increase the yield of soluble antibody fragments (Knappik et al., 1993; Duenas et al., 1994).

In this report we demonstrate that the addition of sucrose to the growth medium inhibits th aggregation of secreted scFv. Depending on the culture conditions, soluble scFv can be found either in the medium or in the bacterial periplasm. A detailed protocol is given for the high-level production of scFv antibody fragments in *E. coli* small-scale cultures and their one-step purification by IMAC.

2. Materials and methods

2.1. Vector constructions

The two vectors used for the expression of scFv antibody fragments are depicted in Fig. 1. The backbone of the plasmids pHOG21 and pHOG31 is derived from Bluescript II SK$^+$ phagemid (Stratagene, La Jolla, CA). To introduce a sequence coding for six histidine residues, a PCR was performed using the plasmid pOPE51 (Kipriyanov et al., 1994) containing scFv phOx31E (Marks et al., 1992) as a template with the sense primer Hu λ1.Mlu (Welschof et al., 1995) and the antisense primer 5'-AAT TCT AGA TTA GTG ATG GTG ATG GTG ATG ACC. The PCR product was blunt ligated into pCR-Script SK(+) (Stratagene) for sequencing and mutagenesis. The substitution of a sequence encoding Cys-Ile

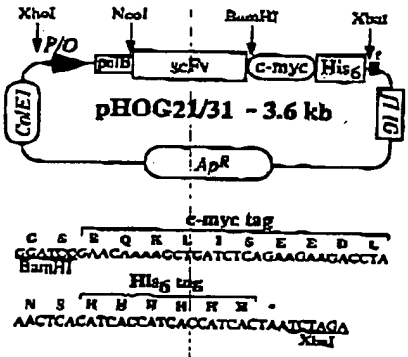

Fig. 1. Schematic representation of the scFv expression vectors. Ap^R, ampicillin resistance-encoding gene; *c-myc*, a sequence encoding an epitope recognized by the monoclonal antibody 9E10; *ColE1*, origin of DNA replication; *f1IG*, intergenic region of phage f1; His_6, a sequence encoding six C-terminal histidine residues; *pelB*, signal peptide sequence of bacterial pectate lyase; P/O, wt *lac* promoter/operator for plasmid pHOG21 and a coliphage T7 promoter P_{A1} combined with two *lac* operators ($P_{A1/04/03}$) for plasmid pHOG31, respectively.

between the c-*myc* epitope and the oligoHis tail for Asn-Ser with the simultaneous removal of a *Bgl*II site was achieved by site-specific mutagenesis according to Kunkel et al. (1987). The DNA ligation of the mutated *Mlu*I-*Xba*I DNA fragment to the large *Mlu*I-*Xba*I fragment of phagemid pSEX81-phOx (Breitling et al., unpublished observations) generated pHOG21-phOx. The plasmid pHOG31 was constructed by substitution of a 214 bp *Xho*I-*Nco*I fragment containing a P_{lac} promoter for a 176 bp *Xho*I-*Nco*I fragment from pSEX (Breitling et al., 1991) comprising a coliphage T7 promoter combined with two *lac* operators ($P_{A1/04/03}$) (Lanzer and Bujard, 1988). The constructed plasmids were used for expression of scFv-phOx recognizing a hapten 2-phenyloxazol-5-one (Marks et al., 1992) and scFv-dmOKT3 specific for the CD3 T cell surface antigen (Kipriyanov et al., unpublished observations).

2.2. Growth conditions for scFv expression

XL1-Blue *E. coli* cells (Stratagene) transformed with the plasmids described above were grown overnight in LB medium with 50 mg/ml ampicillin and 100 mM glucose (LB_{GA}) at 37°C. Dilutions (1/50) of the overnight cultures in LB_{GA} were grown either as 1 ml cultures in 12 ml polystyrene tubes (Greiner, Frickenhausen, Germany) or as 50 ml cultures in 100 ml shake flasks at 37°C with shaking at 280 rpm. When cultures reached $OD_{600} = 0.8$, bacteria were pelleted by centrifugation (1500 × *g*, 10 min, 20°C) and resuspended in an equal volume of fresh LB medium containing 50 mg/ml ampicillin and 0.4 M sucrose. IPTG was added to the desired final concentration and growth was continued at room temperature (22–24°C) or at 16°C for 18–20 h. Control cultures were induced with IPTG without changing the medium. For the preparation of scFv-dmOKT3, the following modifications were made: LB medium was substituted by 2xYT, growth was performed in 1 liter of flask culture and IPTG was added to a final concentration of 0.1 mM.

2.3. Analysis of bacterial samples

Cells were harvested by centrifugation at 5000 × *g* for 10 min and 4°C. The following samples were analysed: (a) supernatant (growth medium clarified by additional centrifugation at 30 000 × *g* for 20–30 min); (b) pelleted intact bacteria; (c) soluble periplasmic content; (d) spheroplast pellet after isolating soluble periplasmic proteins. To isolate soluble periplasmic proteins, the pelleted bacteria were resuspended in 5% of the initial volume of ice-cold 50 mM Tris-HCl, 20% sucrose, 1 mM EDTA, pH 8.0. After a 1 h incubation on ice with occasional stirring, the spheroplasts were centrifuged at 30 000 × *g* for 30 min and 4°C leaving the soluble periplasmic extract as the supernatant and spheroplasts plus the insoluble periplasmic material as the pellet.

SDS-PAGE was performed according to Laemmli (1970) under reducing conditions. Immunoblot analyses using either rabbit serum A (Breitling et al., 1991) recognizing the N-terminus of the processed (without a *pel*B leader) antibody fragment or mouse mAb 9E10 (ICI Biochemicals, Munich, Germany) specific for the c-*myc* oncoprotein were performed as previously described (Kipriyanov et al., 1994). The antigen-binding activity of the scFv-phOx was determined by ELISA performed as previously described (Kipriyanov et al., 1994). Briefly, freshly prepared periplasmic extracts and samples of culture medium were dialyzed into PBS (15 mM sodium phosphate, 0.15 M NaCl, pH 7.4). A phOx-BSA conjugate prepared according to Mäkelä et al. (1978) was used as a specific antigen. Wells coated with BSA alone were used as controls. The concentrations of functional recombinant antibody fragments were determined from the interpolation of their absorbance on standard curves obtained with scFv-phOx purified by antigen-affinity chromatography on phOx-BSA coupled to CNBr-activated Sepharose (Pharmacia, Freiburg, Germany). The yield of scFv-dmOKT3 was determined by comparison of the intensities of the sample bands with those obtained for dilutions of pure scFv-dmOKT3 of known concentration isolated from periplasmic inclusion bodies as previously described (Kipriyanov et al., 1994, 1996a).

2.4. Purification and characterization of scFv-dmOKT3

The cells from 1 liter of a flask culture were harvested by centrifugation as described earlier. The culture supernatant was retained and kept on ice. Soluble periplasmic extracts were obtained from cell

pellets as described above. The culture supernatant and the soluble periplasmic extract were combined, clarified by additional centrifugation (30 000 × g, 4°C, 40 min) and passed first through a glass filter of pore size 10–16 μm and then through a Membrex TF filter of a pore size 0.2 μm (MembraPure, Lörzweiler, Germany). The volume was reduced ten times by concentrating using Amicon YM 10 membranes (Amicon, Witten, Germany). The concentrated supernatant was clarified by centrifugation and thoroughly dialyzed against 50 mM Tris-HCl, 1 M NaCl, pH 7.0 at 4°C. IMAC was performed at 4°C using a 5 ml column of chelating Sepharose (Pharmacia) charged with Ni^{2+} and equilibrated with 50 mM Tris-HCl, 1 M NaCl, pH 7.0 (start buffer). The sample was loaded by passing the sample twice over the column. It was then washed with twenty column volumes of start buffer followed by start buffer containing 50 mM imidazole until the absorbance (280 nm) of the effluent was minimal (about 30 column volumes). Absorbed material was eluted with 50 mM Tris-HCl, 1 M NaCl, 250 mM imidazole, pH 7.0. Fractions corresponding to A_{280} peak were pooled and dialyzed either against 50 mM Tris-HCl, 0.15 M NaCl, 1 mM EDTA, pH 7.4 for size-exclusion FPLC or against PBS for flow cytometric analysis. Protein concentrations were determined by the Bradford dye-binding assay (Bradford, 1976) using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Munich, Germany). The concentration of purified scFv-dmOKT3 was calculated using the extinction coefficient $e^{1\ mg/ml} = 1.836$ at 280 nm calculated according to Gill and von Hippel (1989) using DNAid + 1.8 sequence editor for Macintosh (F. Dardel and P. Bensoussan, Laboratoire de Biochimie, Ecole Polytechnique, Palaiseau, France). Analytical gel filtration of the scFv preparation was performed using a Superdex 75 HR10/30 column (Pharmacia). Sample volume and flow rate were 200 μl and 0.5 ml/min, respectively. A low molecular weight gel filtration calibration kit (Pharmacia) was used to calibrate the column.

2.5. Flow cytometry

5×10^5 $CD3^+$ Jurkat or $CD3^-$ JOK-1 cells in 5 μml RPMI 1640 medium (Gibco BRL, Eggenstein, Germany) supplemented with 10% fetal calf serum (FCS) and 0.1% sodium azide (referred to as complete medium) were incubated with 100 μl of scFv (100 μg/ml) for 45 min on ice. The cells were washed with complete medium and incubated with 100 μl of 10 μg/ml anti-c-*myc* mAb 9E10 (ICI Biochemicals) in the same buffer for 45 min on ice. The cells were washed again and incubated with 100 μl of FITC-labeled goat anti-mouse IgG (Gibco BRL, Eggenstein, Germany) under the same conditions as before. The cells were then washed again and resuspended in 100 μl of 1 μg/ml solution of propidium iodide (Sigma, Deisenhofen, Germany) in complete medium to exclude dead cells. The relative fluorescence of stained cells was measured using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA).

3. Results

3.1. Effect of sucrose and incubation conditions on the amount and location of soluble scFv

It has been shown that the addition of 0.4 M sucrose to the minimal culture medium of *E. coli* transformants carrying the gene of β-lactamase cloned downstream of the P_{tac} promoter and ompA signal sequence increases the accumulation of soluble β-lactamase (Bowden and Georgiou, 1990) in the bacterial periplasm. To achieve a maximal yield of soluble scFv, we therefore studied the influence of sucrose together with various growth and induction conditions using *E. coli* cells grown in rich medium. For these experiments, we constructed the vector pHOG21 (Fig. 1) containing the wt *lac* promoter that is about three times weaker than P_{tac} (Lanzer and Bujard, 1988). As a model antibody, we used scFv-phOx that recognizes the hapten 2-phenyloxazol-5-one (Marks et al., 1992).

We have found that adding sucrose to the growth medium significantly increases the yield of secreted soluble scFv. The location of secreted recombinant antibody (bacterial periplasm or culture medium) was dependent on the cultivation conditions (Table 1). For example, bacterial cells induced overnight in the presence of sucrose and incubated in shake-flasks, were found to release substantial amounts of scFv-phOx into the medium (Table 1). The ratio between

the amounts of soluble scFv in the periplasm and in the medium was dependent on the concentration of the inducer; at higher IPTG concentrations smaller amounts of antibody fragments were isolated from periplasm (Table 1). In contrast, incubation in shake-tubes of the same bacterial starting cultures grown in the same medium containing sucrose and IPTG did not lead to the release of scFv into the medium. The quantitative data given in Table 1 indicate that the addition of sucrose to the medium results in a 15–25-fold increase of soluble scFv-phOx production for bacterial shake-tube cultures and an 80–150-fold increase for shake-flask cultures. Unlike the previously described experiments where β-lactamase was expressed under the control of the P_{tac} promoter in *E. coli* cells grown in minimal medium (Bowden and Georgiou, 1990), we observed the positive effect of sucrose only when it was added just prior to the induction of *lac* promoter (data not shown).

3.2. Effect of temperature and promoter strength on the production of soluble scFv

We have found that scFv derived from combinatorial antibody libraries displayed on the surface of filamentous phage (phAbs) often appear to be more soluble than scFv derived from hybridoma cells (unpublished results). It seems that the phAbs may be selected not only for antigen binding but also for solubility. Therefore, instead of using the library derived scFv-phOx, for further experiments we used scFv-dmOKT3 consisting of the V_H and V_L domains of monoclonal antibody OKT3 (Van Wauwe et al., 1980) specific for the human CD3 T cell surface antigen (Kipriyanov et al., unpublished observations). Incubation of induced bacteria harboring the plasmid pHOG21-dmOKT3 in the presence of 0.4 M sucrose showed exactly the same picture as we observed for scFv-phOx, namely accumulation of soluble scFv in the periplasm following shake-tube incubation and release of antibody fragments into the medium when cells were grown in shake-flasks (Table 1). The total yield of soluble scFv-dmOKT3 obtained was comparable with that for scFv-phOx.

Lowering the bacterial growth temperature has been shown to decrease periplasmic aggregation and increase the yield of soluble antibody protein (Skerra and Plückthun, 1991; Plückthun, 1994). We therefore incubated bacteria induced with 0.1 mM IPTG in shake-flasks with medium and sucrose at 16°C. Under these conditions, the release of scFv into the medium was substantially decreased and most of the soluble scFv were found in the periplasm (data not shown). However, the yield of soluble antibody fragments was 2–3 times lower than the total amount of scFv in the periplasm and medium of bacterial cultures induced at room temperature.

To test the effect of increasing the promoter strength under the above conditions, we cloned the scFv-dmOKT3 gene into plasmid pHOG31, which has a coliphage T7 promoter P_{A1} combined with two *lac* operators ($P_{A1/04/03}$) instead of P_{lac}. The $P_{A1/04/03}$ promoter has been shown to be about six times stronger in vivo than P_{lac} (Lanzer and Bujard, 1988). Immunoblot analysis of the soluble and insoluble fractions of cells incubated in shake-tubes and induced at room temperature with different IPTG concentrations in the presence of sucrose demonstrated that increasing the promoter strength led to a higher intracellular accumulation of scFv, but mainly in an insoluble form (Fig. 2). The amounts of soluble antibody fragments found in the periplasm were higher for plasmid pHOG21 containing the weaker

Table 1
Production levels of soluble scFv antibody fragments in *E. coli* under different conditions of induction [a]

Growth vessels	IPTG	Sucrose	Yield of soluble scFv (mg/l of culture) [b]		
	mM		Periplasm	Medium	Total
scFv-phOx					
Flasks	0.1	No	0.08	< 0.02	0.10
		Yes	0.8	14.5	15.3
	1.0	No	0.18	< 0.02	0.20
		Yes	0.3	16.2	16.5
Tubes	0.1	Yes	2.5	< 0.02	2.5
	1.0	Yes	3.0	< 0.02	3.0
scFv-dmOKT3					
Flasks	0.1	Yes	0.2	10.0	10.2
Tubes	0.1	Yes	3.5	< 0.02	3.5

[a] For all experiments, induction was performed at room temperature.

[b] For scFv-phOx calculations were made on the basis of ELISA data, for scFv-dm OKT3 – on the basis of immunoblot analyses (see Section 2).

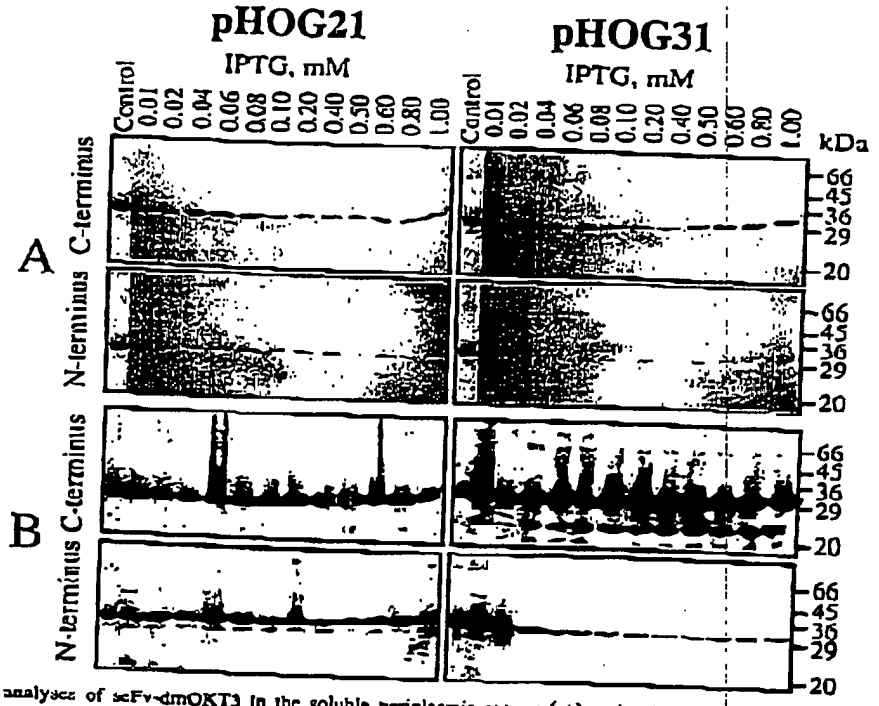

Fig. 2. Immunoblot analyses of scFv-dmOKT3 in the soluble periplasmic extract (*A*) and spheroplast pellet (*B*) of shake-tube *E. coli* cultures harboring plasmids pHOG21 and pHOG31 after induction with different IPTG concentrations. ScFv were detected using either monoclonal antibody 9E10 recognizing the C-terminal c-*myc* epitope (C-terminus) or serum A (Breitling et al., 1991) recognizing the N-terminus of the processed antibody fragment (N-terminus). All loaded samples correspond to 75 μl of culture. As a control, 1 mg of pure scFv-dmOKT3 isolated from inclusion bodies (Kipriyanov et al., 1996a) was used. The positions of molecular mass markers are shown on the right.

P_{lac} promoter and seemed to be fairly independent of the inducer concentration (Fig. 2A). Analysis of the spheroplast pellet after extracting the soluble periplasmic proteins showed that both plasmids produced large amounts of insoluble scFv aggregates (inclusion bodies). In case of plasmid pHOG21, inclusion bodies were formed in the periplasm by aggregation of processed scFv (without the *pelB* leader sequence) which is recognized by serum A specific for the N-terminus of processed antibody fragments (Breitling et al., 1991). In contrast, for *E. coli* cells harboring plasmid pHOG31 and induced with IPTG concentrations higher than 40 mM, aggregation of non-processed cytoplasmic scFv precursor predominated (Fig. 2B). In the latter case, increasing the IPTG concentration also caused the appearance and accumulation of scFv degradation products (Fig. 2B).

3.3. Purification and characterization of scFv-dmOKT3

The above findings were used for the preparation of pure scFv-dmOKT3 specific for the CD3 T cell surface antigen. The supernatant and periplasmic content of an induced culture XL1-Blue/pHOG21-dmOKT3 grown overnight at room temperature in the presence of 0.4 M sucrose was concentrated and passed through a Ni^{2+} charged Chelating Sepharose column. After washing the column with buffer containing 50 mM imidazole, the bound scFv was eluted

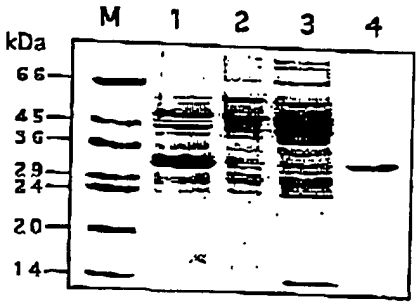

Fig. 3. 12% SDS-PAGE analysis of scFv-dmOKT3 at different steps of purification. Lanes: M, molecular mass markers (values in kDa are shown on the left); 1, total cell lysate; 2, soluble periplasmic content; 3, concentrated culture medium; 4, scFv purified by IMAC. The gel was stained with Coomassie Brilliant Blue.

with 250 mM imidazole as a single peak in 2.5 column volumes. We routinely obtained 3–4 mg of scFv from 1 liter of bacterial culture with a purity of about 95% (Fig. 3). Analytical gel filtration demonstrated that the isolated scFv consisted mainly of monomers (Fig. 4). The ability of the purified anti-CD3 scFv to bind its target molecule was shown by flow cytometry on CD3-positive Jurkat T cells (Fig.

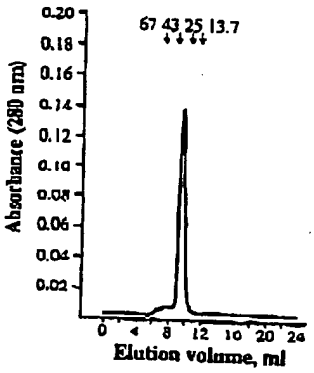

Fig. 4. Analytical gel filtration of purified scFv-dmOKT3 on a Superdex 75 column. The elution buffer was 50 mM Tris-HCl, 0.15 M NaCl, 1 mM EDTA, pH 7.4. Sample volume and flow rate were 200 μl and 0.5 ml/min, respectively. Molecular masses were calibrated with BSA (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (13.7 kDa).

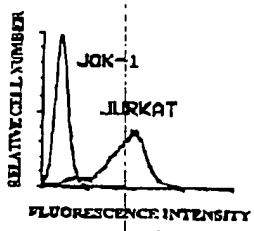

Fig. 5. Flow cytometric analysis of scFv-dmOKT3 binding to CD3-positive Jurkat cells and CD3-negative JOK-1 cells.

5). In a parallel experiment, no binding to CD3-negative B cell line JOK-1 was detected.

4. Discussion

The *E. coli* secretion machinery directs recombinant scFv proteins to the periplasm. At least some fraction of secreted recombinant antibody fragments folds into its native state. It is now clear that the degree of successful folding depends on the primary sequence of the variable domains (Plückthun, 1994; Knappik and Plückthun, 1995). Some antibody fragments appear to perturb the integrity of the outer membrane and are released into the medium (Takkinen et al., 1991; Sizmann et al., 1993). This membrane leakiness, whose molecular cause is still unknown, also seems to depend on the primary sequence of the antibody. For example, in identical *E. coli* host/vector systems, 1–5 mg/l of anti-glycophorin A 1C3 scFv was found in the culture medium (Lilley et al., 1994) compared to 0.1–0.5 mg/l of anti-DNP U7.6 scFv and no anti-transferrin receptor OKT9 scFv (George et al., 1994). Therefore, protein engineering was recently proposed as a strategy to obtain correctly folded periplasmic proteins in high yield (Knappik and Plückthun, 1995). However, this approach is expensive and can therefore only be used for a few of the most important antibodies and is not feasible when screening a large number of binding candidates.

The aggregation of recombinant proteins in the *E. coli* periplasm can be reduced by growing the cells in the presence of certain sugars, e.g. raffinose or sucrose (Bowden and Georgiou, 1990). These sugars are small enough to diffuse into the periplasmic

space of *E. coli* but they are not metabolized. Due to the accompanying increase in osmotic pressure, it is possible that the periplasmic space is enlarged. This would effectively decrease the local concentration of secreted protein, resulting in less aggregation (Kiefhaber et al., 1991). If this hypothesis is true then some release of soluble periplasmic protein through a perturbed outer membrane could effect the competition between polypeptide folding and aggregation, leading to an increase in the total yield of soluble antibody fragments.

In this report, we demonstrate that induction of bacterial shake-tube cultures in the presence of 0.4 M sucrose gives a 15–25-fold increase in the yield of soluble scFv production in the *E. coli* periplasm. Simply changing the incubation conditions by using shake-flasks causes further release of scFv into the medium and leads to an additional 3–5-fold increase of soluble recombinant antibody production. We observed a similar picture for a number of scFv antibody fragments of different specificity. The conditions could be chosen for the preferential location of the soluble antibody fragments e.g. in the medium for the isolation of relatively large amounts of scFv that could be purified in a one-step procedure or in the periplasm for the rapid screening of antibody binding characteristics (Kipriyanov et al., 1996b).

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